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Concanavalin A-Immobilized Polystyrene Nanospheres Capture HIV-1 Virions and gp120: Potential Approach Towards Prevention of Viral Transmission

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To establish an effective tool for the prevention of HIV-1 transmission, lectin-immobilized polystyrene nanospheres were synthesized and examined for their HIV-1 capture activity. When concanavalin A (Con A) was immobilized on the surface of polystyrene nanospheres (400 nm in diameter) with poly(methacrylic acid) branches and incubated with HIV-1 suspension at room temperature for 60 min, the nanospheres (Con A-NSs) achieved a >3.3 log and a 2.2 log reduction of viral infectivity in HIV-1 (III_B strain) suspension at a concentration of 2 and 0.5 mg/ml, respectively. Meanwhile, Con A-free nanospheres, which were not immobilized with Con A, achieved only a 0.29 log reduction at 0.5 mg/ml. Con A-NSs (2 mg/ml) could also reduce the gp120 level of III_B and HE strains to <7.1% and 5.5% of each control, respectively. The combination of Con A-NS treatment followed by filtration with a microporous membrane efficiently removed virion-free gp120 as well as infectious viral particles from HIV-1 suspension. Electron microscopic examination demonstrated that HIV-1 virions were trapped on the surface of Con A-NSs. Thus, Con A-NSs can capture HIV-1 virions and gp120 with high affinity, and may have potential as an effective tool for the prevention of HIV-1 transmission. ***J. Med. Virol. 56:327–331, 1998.***

KEY WORDS: AIDS; lectin; glycoprotein; antiviral

INTRODUCTION

Progress of combination chemotherapy with HIV-1 reverse transcriptase and protease inhibitors has achieved long-sustained suppression of viral replication in HIV-1-infected individuals, leading to widespread optimism that AIDS will become a manageable disease [Deeks et al., 1997]. However, considering the high cost and low compliance of long-term combination chemotherapy, establishment of an effective tool for the prevention of HIV-1 transmission is extremely desirable. Since a major route of HIV-1 infection is via heterosexual intercourse, reduction of viral infectivity in the vagina by microbicides or other substances seems to be an attractive approach to the prevention of HIV-1 transmission [Potts, 1994]. Several compounds are possible candidates. Nonoxynol-9 is a spermicide that has shown a protective effect against HIV-1 infection [Feldblum and Weir, 1994]. Another group has demonstrated the inhibitory effect of the anti-HIV-1 polyanion PAVAS (a copolymer of acrylic acid with vinylalcohol) on intracutaneous and intravaginal herpes virus infection in mice [Neyts and De Clercq, 1995]. However, the efficacy and toxicity of both nonoxynol-9 and PAVAS in humans have not been fully elucidated yet.

We recently developed a novel technology for polystyrene nanospheres having hydrophilic polymer chains with functional groups [Riza et al., 1995; Chen et al., 1996]. These nanospheres can be used as immunolatex when certain antibodies are introduced into their surfaces. The peptide drug calcitonin also shows pharmaceutical activity after oral administration when

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it has been physically immobilized onto polystyrene nanospheres coated with water-soluble polymer chains [Sakuma et al., 1997]. HIV-1 is an enveloped virus with a diameter of 100 nm and expresses gp120 and gp41 antigens on its envelope [Evans and Levy, 1993]. Since gp120 is a heavily mannosilated glycoprotein, it interacts strongly with some lectins [Lifson et al., 1986; Balzarini et al., 1991]. In particular, concanavalin A (Con A) has high affinity with gp120, assuming that gp120 and virions will be effectively captured by Con A when they are ideally immobilized on the surface of nanospheres. This prompted us to synthesize Con A-immobilized polystyrene nanospheres (Con A-NSs) and examine their HIV-1 capture activity in vitro. In this study, we will show that Con A-NSs can capture HIV-1 virions and gp120, and thereby infectivity is significantly reduced in viral suspension.

MATERIALS AND METHODS

Nanospheres

The synthetic procedures for polystyrene nanospheres having poly(methacrylic acid) chains were prepared by the copolymerization of styrene with poly(*tert*-butylmethacrylate) and the following acid hydrolysis were described previously [Riza et al., 1995]. Con A, which was purchased from Sigma Chemical Co. (St. Louis, MO), was stably immobilized on the surface of polystyrene nanospheres by chemical reactions between the carboxyl group of poly(methacrylic acid) and the amino group of Con A, according to a method described elsewhere [Akashi et al., 1998]. The nanospheres were thoroughly washed with HEPES buffer (pH 7.4) to remove unimmobilized Con A and measured for their mean diameter (400 nm) and the amount of immobilized Con A (0.91 $\mu g/cm^2$). The Con A-NSs were suspended in the buffer and stored at 4°C until use.

Viruses

Three different strains of HIV-1 (III_B, HE, and $III_{Ba\text{-}L}$) were used in the experiments. The HE strain is a T-cell-tropic clinical isolate from a Belgian patient with AIDS [Pauwels et al., 1990], while the $III_{Ba\text{-}L}$ strain is a macrophage-tropic HIV-1 [Perno et al., 1988]. The III_B and HE strains were propagated in MOLT-4 cells. The infected cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics (culture medium), and their culture supernatants were centrifuged at 3,000 rpm for 10 min, filtrated, and stored at −80°C until use. The infectivity of viral stocks was determined in MT-4 cells, and their gp120 antigen level was measured by a gp120 antigen-capture ELISA kit (Advanced Biotechnologies, Columbia, MD), respectively. The $III_{Ba\text{-}L}$ strain was propagated in peripheral blood mononuclear cells, which were obtained from healthy donors and stimulated with phytohemagglutinin, as previously described [Baba et al., 1994].

HIV-1 Capture Assays

The suspension of HIV-1 (0.5 ml) was mixed with an equal volume of various concentrations of Con A-NSs and incubated at room temperature. After a 60-min incubation, the suspension was centrifuged at 8,200*g* for 10 min. The supernatants of samples were examined for their gp120 antigen level and viral infectivity. Viral infectivity was assessed by microscopic observation for HIV-1-induced cytopathicity in MT-4 cells and expressed as a 50% cell culture infectious dose per milliliter ($CCID_{50}$/ml). The pellets of centrifuged nanospheres were subjected to a scanning electron microscopic examination. As comparison, Con A-free nanospheres that were not immobilized with Con A were used in all experiments.

Electron Microscopy

Con A-NSs were incubated with HIV-1 suspension and centrifuged as described above. For scanning electron microscopy, the pellets of nanospheres were washed with phosphate-buffered saline (PBS) to remove untrapped viral particles and fixed with 1% OsO_4 at 4°C for 60 min. Then the samples were dehydrated with escalating concentrations of ethanol (50–99.5%) and freeze-dried. Scanning was carried out at 5 kV with a Hitachi S-4100H electron microscope (Tokyo, Japan). For transmission electron microscopy, the pellets of nanospheres were subjected to immunostaining with a murine anti-gp120 HIV-1 monoclonal antibody conjugated to colloidal gold (Immunodiagnotics, Bedford, MA). The samples were washed extensively with PBS, fixed with 2.5% glutaraldehyde and 1% OsO_4, and dehydrated with ethanol. They were examined with a Hitachi H-700H electron microscope at 200 kV.

RESULTS

When the suspension of HIV-1 (III_B strain) was incubated in the presence of various concentrations of Con A-NSs and examined for infectivity, the titers of viral suspension incubated with 0, 0.5, and 2 mg/ml Con A-NSs were 370,000, 2,200, and < 200 $CCID_{50}$/ml, respectively (Table I). Consequently, Con A-NSs achieved a >3.3 log and a 2.2 log reduction of viral infectivity at 2 and 0.5 mg/ml, respectively. Even at a concentration of 0.13 mg/ml, Con A-NSs reduced infectivity to 15% of control level (Table 1). Interestingly, Con A-free nanospheres, which were not immobilized with Con A, could also reduce the infectivity of HIV-1, yet the activity was not comparable (51% at 0.5 mg/ml) to that of Con A-NSs (Table I).

Since the viral suspension contained not only HIV-1 particles but also virion-free gp120 antigen, we examined whether Con A-NSs could reduce the total gp120 level. A dose-dependent reduction of gp120 was observed in the viral suspension with increasing concentrations of Con A-NSs, irrespective of the HIV-1 strains examined (Fig. 1). The nanospheres could decrease the gp120 level of the III_B strain (Fig. 1A) and the HE strain (Fig. 1B) to <0.30 and 0.33 ng/ml at a concentra-

TABLE I. Effect of Con A-NS Treatment on HIV-1 Infectivity*

Nanosphere	Concentration (mg/ml)	Infectivity[a] ($\times 10^4$ $CCID_{50}$/ml)	%
Con A-immobilized	2	<0.02	<0.05
	0.5	0.22 ± 0.09	0.59
	0.13	5.6 ± 1.4	15
Con A-free[b]	0.5	19 ± 7	51
None	0	37 ± 14	100

*Data represent mean values ± SD for triplicate samples.
[a]The infectivity of HIV-1 (III_B strain) was determined by microscopic observation for virus-induced cytopathicity in MT-4 cells.
[b]Nanospheres not immobilized with Con A.

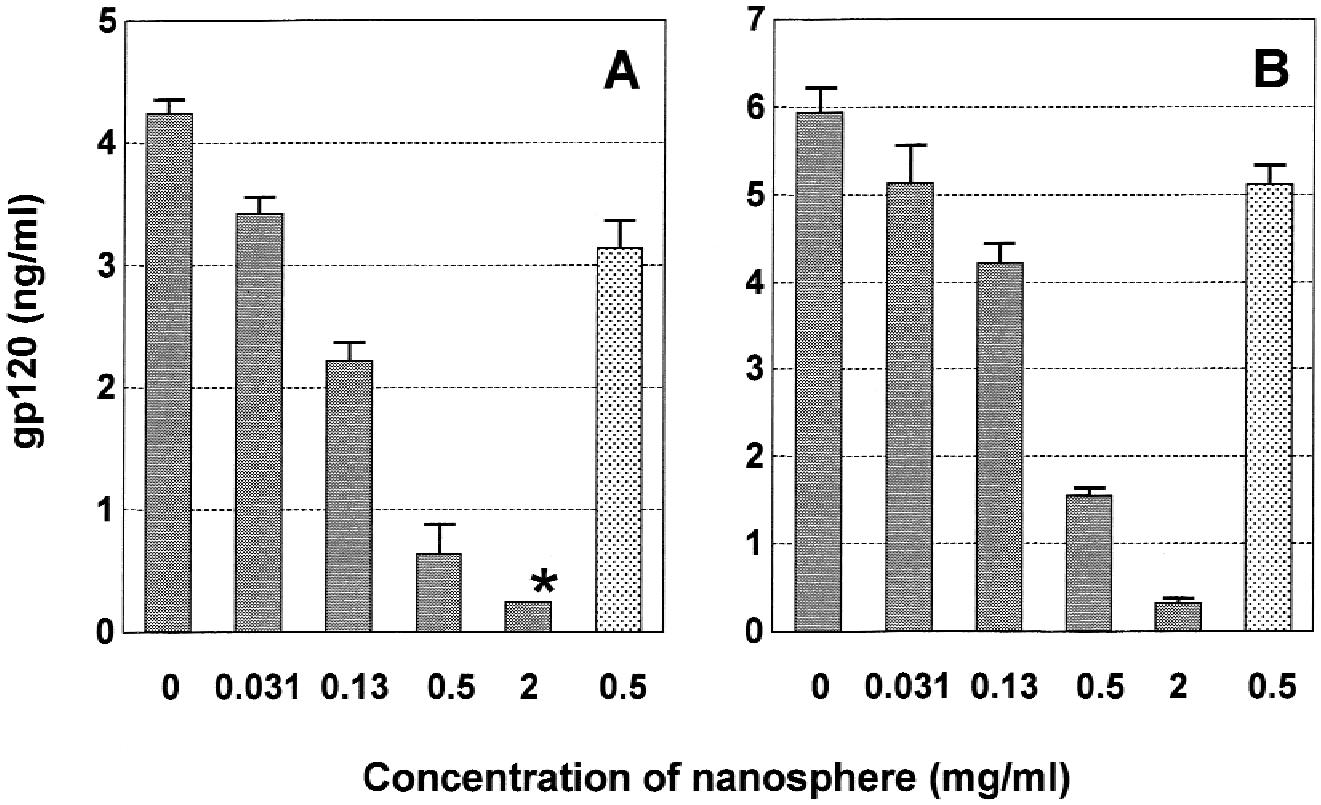

Fig. 1. Effect of Con A-NSs on HIV-1 gp120. The suspension of III_B **(A)** or HE **(B)** strain was mixed with various concentrations of Con A-immobilized (solid column) or Con A-free (dotted column) nanospheres and incubated at room temperature. After a 60-min incubation, the mixture was centrifuged, and the supernatants were examined for their gp120 antigen level. *Level of gp120 in the presence of 2.0 mg/ml Con A-NSs was below the detection threshold of the ELISA kit (0.3 ng/ml). Data represent mean values ± SD for triplicate samples.

tion of 2 mg/ml, respectively. These values are <7.1% and 5.5% of their control level. Similar to the result for viral infectivity (Table I), Con A-free nanospheres could slightly reduce the gp120 level of both strains at a concentration of 0.5 mg/ml (Fig. 1A,B). Furthermore, the macrophage-tropic HIV-1 strain $III_{Ba\text{-}L}$ was also captured by Con A-NSs. Approximately 63% of the total gp120 (1.3 ng/ml) was trapped by the nanospheres at 0.5 mg/ml. When the infectivity of $III_{Ba\text{-}L}$ was measured by a focus formation assay using HeLa cells that expressed CD4 and CCR5 on their surface (MAGI-CCR5) [Chackerian et al., 1997], Con A-NS achieved 50% reduction of viral infectivity at this concentration (data not shown).

Electron microscopy was conducted to determine whether HIV-1 particles were trapped on the surface of Con A-NSs. When Con A-NSs were treated with HIV-1 suspension, a few spherical particle-like structures, approximately 100 nm in diameter, were bound to the surface of nanospheres 400 nm in diameter (Fig. 2A). No such structures were identified on the surface of Con A-NSs treated with culture medium (Fig. 2B). Immunostaining with a colloidal gold-conjugated anti-gp120 monoclonal antibody and transmission electron microscopy revealed that viral particles and gp120 antigens were trapped on the surface of Con A-NS treated with HIV-1 suspension (Fig. 2A, lower right). No colloidal gold conjugation was observed for the Con A-NS incubated with the culture supernatant of uninfected MOLT-4 cells (Fig. 2B, lower right).

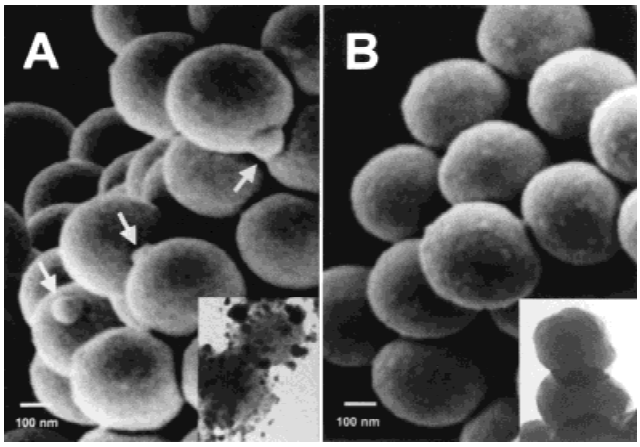

Fig. 2. Scanning electron microscopy for Con A-NSs. The nanospheres were incubated with HIV-1 suspension **(A)** or culture medium **(B)** and centrifuged. The pellets were washed with PBS to remove untrapped viral particles and fixed with 1% OsO_4. The samples were dehydrated and freeze-dried in the routine manner. Scanning observation was carried out at 5 kV with a Hitachi S-4100H electron microscope (original magnification ×20,000). At lower right in both A and B is the photograph of transmission electron microscopy of Con A-NS after immunostaining with a colloidal gold-conjugated anti-gp120 monoclonal antibody. In this experiment, the nanospheres were incubated with HIV-1 suspension (A) or the culture supernatant of uninfected MOLT-4 cells (B). The samples were washed extensively with PBS, fixed with 2.5% glutaraldehyde and 1% OsO_4, and dehydrated with ethanol. They were observed with a Hitachi H-700H electron microscope at 200 kV.

A microporous membrane filter with an appropriate pore size is an effective tool for removal of HIV-1 particles from various aqueous samples [Hamamoto et al., 1989]. In fact, PLANOVA 75™ (Asahi Chemical Industry Co., Tokyo, Japan), a commercially available virus removal filter with a mean pore size of 75 nm, achieved almost complete removal of viral particles from HIV-1 suspension (Table II). However, the filter could not remove virion-free gp120 from the suspension because of its much smaller size than the filter pore size. Only 40% of the total gp120 (5.0 ng/ml) seemed to be trapped by the filter, suggesting that the trapped gp120 (2.0 ng/ml) was virion-associated. When HIV-1 suspension was treated with Con A-NSs and then filtrated, the remaining gp120 of the filtrated suspension resulted in 0.19 ng/ml, which was only 3.8% of the total gp120 (Table II). These results indicate that the combination of Con A-nanosphere treatment and filtration may have practical usefulness, since it can remove HIV-1 particles and gp120 from viral suspension without centrifugation.

TABLE II. Removal of HIV-1 gp120 by Combination Treatment With Con A-NSs and Microporous Membrane Filter (PLANOVA 75™)*

Treatment	gp120 (ng/ml)	Infectivity ($\times 10^4$ $CCID_{50}$/ml)
None	5.0 ± 0.2	44
Filtration only	3.0 ± 0.2	<0.02
Filtration + Con A-NSs[a]	0.19 ± 0.04	<0.02

*A commercially available virus removal filter with a mean pore size of 75 nm. Data represent mean values ± SD for triplicate samples.
[a]0.5 mg/ml.

DISCUSSION

We have shown that Con A-NSs efficiently capture HIV-1 particles and gp120 antigen, resulting in significant decrease of the infectivity of viral suspension. Furthermore, virion-free gp120 can be removed from certain contaminated materials when combined with filtration. A similar approach has been undertaken by another group with a polyanion-immobilized porous membrane (Yamashita et al., personal communication). This approach is based on the fact that polyanions, such as dextran sulfate and PAVAS, have high affinity with the gp120 of HIV-1 and inhibit viral adsorption to the host cells [Baba et al., 1990; Schols et al., 1990]. However, it has been demonstrated that the anti-HIV-1 activity of polyanions depends on the host cell type and on the V3 loop of gp120, and that dextran sulfate does not inhibit the replication of macrophage-tropic strains of HIV-1 [Meylan et al., 1994]. Thus, the polyanion-immobilized porous membrane does not seem to efficiently remove HIV-1 virions from a variety of clinical samples. Since lectins, including Con A, recognize the oligosaccharide chain of gp120 instead of the V3 loop, Con A-NSs also interact with the gp120 of macrophage-tropic viruses and remove them from viral suspension, indicating an advantage of our nanospheres over the polyanion-immobilized porous membrane. Furthermore, the virions captured by Con A-NS were still infective, but their infectivity appeared to be <5%, compared with that of the original HIV-1 suspension (data not shown).

Another advantage of Con A-NSs is that they are extremely small particles (approximately 400 nm in diameter) and can be prepared as a suspension in various buffers. We estimated that the number of nanospheres was approximately 4×10^{10} particles per milligram, and that their surface area reached 200 cm^2. Although the capture efficiency of nanospheres depends on the amount of immobilized Con A, the surface of nanospheres used in this study appeared to be fully covered with Con A (0.91 $\mu g/cm^2$). We assume that the suspension of Con A-NSs may also be applicable as a topical agent for prophylaxis in HIV-1 infection.

Several issues, including efficacy, specificity, and toxicity in vivo, remain to be elucidated before Con A-NSs are introduced into human trials. Unlike anti-gp120 antibodies, the interaction between Con A and gp120 is not specific. Therefore, it is possible that the presence of semen or vaginal fluid affects the Con A-gp120 interaction. Recent preliminary experiments demonstrated that the capture activity of Con A-NSs was considerably impaired by the presence of semen (data not shown). However, an appropriate selection of immobilized lectins might increase the specificity of nanospheres for HIV-1 and could circumvent this problem. In fact, the nanospheres immobilized with *Lens culinaris agglutinin* and *Galanthus nivalis lectin,* which are more specific to mannose than Con A, also

displayed a comparable capture activity, even though the amount of immobilization was lower than that of Con A [Akashi et al., 1998].

Con A is known to have various toxicities in vivo [Tiegs et al., 1992; Bento et al., 1997; Nishida et al., 1996]. At present, the in vivo toxicity of Con A-NSs is unknown. The nanospheres should be used topically or ex vivo. However, their toxic side effects on skin or mucous membrane need to be clarified, and this is now under investigation. We assume that the Con A-NSs are less toxic than free (unimmobilized) Con A, since it is stably immobilized on the surface of polystyrene nanospheres by chemical reactions and may not be detached from the surface of nanospheres.

In conclusion, the potent capture activity of Con A-NSs for HIV-1 particles and gp120 was demonstrated. Consequently, HIV-1 particles and gp120 can be removed from viral suspension by a 60-min incubation at room temperature followed by centrifugation or filtration.

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